THE FLOCCULATION OF BACTERIA.

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Small particles of clay or finely divided chemical precipitates may remain suspended in water for a long time. When, however, certain salts are added to the water, the microscopic particles are seen to settle to the bottom of the liquid in which they were formerly suspended, with greater or less rapidity according to the kind of salt, the amount of salt per volume of liquid, and the temperature. In a previous paper* I have shown that the suspension of the particles is caused by the molecular pressure of the individual water molecules upon the surfaces of the suspended solid, with the result that there is, as it were, a hydrate formed. By reason of its superior attraction for water, the saline flocculating agent causes the withdrawal of the water molecules which had crowded upon the surfaces of the particles. What was formerly a surface pressure, now becomes a surface tension, which, being exerted upon all the particles, causes them to run together into little clumps which quickly gravitate to the bottom of the liquid.

The flocculating action is to be traced chiefly to the metallic portion of the salt, and in a small degree only to the acid radicle. The metals vary in the intensity of their action, some being strong, others weak; as an instance, calcium is about 160 times more powerful than potassium. These agents not only cause the coagulation of particles which are visible with the microscope, but they also induce substances which are in solution to precipitate. The latter are in what is called "pseudo-solution"—that is, they consist of molecular aggregates which are just retained in solution and no more. The flocculating agent induces a further coalescence of the molecular aggregates, and these being no longer able to remain in solution separate out as a precipitate.

If we look upon bacteria growing in a culture fluid as particles in suspension, it seems reasonable to hope that they might be sensitive to the action of flocculating agents precisely like particles

* Journ. Soc. Chem. Industry, xvi. 872; xvii. 117.

of clay. If they behave like suspended particles, flocculation may be utilised as a means of obtaining them more readily from the solutions in which they have been grown. But better than this, flocculation as in pseudo-solution might be the means of separating those bacteria which are supposed to be ultra-microscopical—as, for example, the organism of pleuro-pneumonia (Nocard and Roux), which is hardly visible under the highest powers of the microscope. It might also lead to the elucidation of some questions connected with the agglutination of bacteria by active sera.

With regard to the choice of flocculating agents, it must be borne in mind that salts of the heavy metals would coagulate the constituents of the culture media and of the organism. We are, therefore, deprived of the strongest agents. The salts of the zinc and iron metals are too prone to form basic salts, and accordingly a choice of the metals of the alkalies and alkaline earths remain. Of these metals, calcium has the highest flocculating power, and, therefore, calcium chloride was employed in my experiments. The density of bacteria as shown by Almquist is about 1·4, and that of most inorganic particles much more—say from 1·8 to 2·6; it was, therefore, to be expected that a strong flocculating agent would be required.

Preliminary experiments showed that a flocculation was obtained with calcium chloride, and also indicated what should be the approximate strength of the flocculating solution. At first a meat-extract, neutralised with sodium hydrate to phenolphthalein, and at a later period meat bouillon, neutralised in the same way, were used for growing the cultures. The organisms which I regarded as being typical for the purpose were *Bact. prodigiosum*, *Bact. coli commune*, and *Bact. typhi*. A solution of calcium chloride crystals was prepared of such strength that a litre contained the molecular weight expressed in grams, *i.e.*, the solution contained 40 grams of calcium per litre. Dilutions of this were made in ratios of $\frac{1}{10}$, $\frac{1}{25}$, and $\frac{1}{50}$. The precipitation of the cultures was conducted in small test-tubes, into which were pipetted 2 c.c. of the cultures which had generally grown for two days at 30° C., and had been passed through a cotton-wool plug to eliminate clumps. The quantities of the flocculating agents that were added varied generally from 0·2 to 1·0 c.c. I may

mention that testing by means of the hanging drop is not advisable when using lime salts, unless the platinum loop is cleaned in hydrochloric acid after each ignition.

Precipitation of bacteria by calcium chloride.—The results will probably be best seen by looking at the following table, in which the relative precipitation is indicated by numbers running from 1 to 6; 1 represents uniform turbidity with a very slight precipitate, whilst 6 indicates a complete precipitation with a clear supernatant liquid. The intermediate numbers represent intermediate stages of precipitation. A zero means that no change occurred, while a plus shows that flocculation was visible when the liquid was examined with a hand lens:—

c.c. Calcium chloride added to 2 c.c. suspension.		Calcium chloride expressed as milligrams of calcium per 100 c.c. of total suspension.		*B. prodigiosum.*		*B. coli commune.*		*B. typhi.*	
				½ hour.	1 hour.	½ hour.	1 hour.	½ hour.	1 hour.
0·2	$\frac{1}{10}$ gram-molecule.		36	0	1	2	3	4	6
0·4			67	2	4*	2	3	4	6
0·6			92	3	5†	2	4*	4	6
0·8			114	3	5	3	5†	5	6
1·0			133	3	5	3	5	5	6
0·2	$\frac{2}{25}$ gram-molecule.		14			1	2	—	—
0·4			27			1	2	5	6
0·6			37			1	2	5	6
0·8			46			2	3	—	—
1·0			53			2	3	—	—
0·2	$\frac{1}{50}$ gram-molecule.		7					0	0
0·4			13					0	+
0·6			18					+	4*
0·8			23					3	5†
1·0			27					5	6

It will be seen that the bacteria exhibit different susceptibilities to the precipitating action of calcium chloride. *Bact. typhi* is more sensitive than *Bact. prodigiosum*, and the latter than *Bact.*

coli commune. The bacteria were thoroughly precipitated, and presented similar appearances with the following amounts of calcium in milligrams per 100 c.c. of total suspension (marked with a † in the table).

Bact. typhi	23
„ *prodigiosum*	92
„ *coli commune*	114

A proportion roughly as 1 : 4 : 5.

Bearing in mind the utility of being able to separate the organisms quickly by filtration through paper, I filtered the cultures through paper of fine texture like that used for fine precipitates such as barium sulphate. To avoid the possible passage of the bacteria over the inside and down the outside of the filter, the margin of the paper was painted with vaseline. The smallest quantities of calcium necessary to give a clear filtrate were as follows (marked with a * in table) :—

Bact. typhi	18
„ *prodigiosum*	67
„ *coli commune*	92

A proportion again roughly as 1 : 4 : 5.

With the object of ascertaining whether or not the bacteria were retained on the filter paper, the *Bact. prodigiosum* precipitate was washed on the filter, five times, successively with dilute calcium chloride, using 2 c.c. portions of $\frac{1}{100}$ gram-molecule per litre solution. The number of bacteria were determined in a large loop of the successive washings by means of plate cultures. The filtrate contained many bacteria; the fourth washing grew two colonies, and the fifth washing was sterile. The bacteria that were precipitated had been retained upon the filter paper. It is, therefore, shown that practically complete coagulation of a meat or bouillon culture can be obtained in an hour by the addition of 0·6 c.c. $\frac{1}{10}$ gram-molecule per litre solution of calcium chloride to 2 c.c., or 0·66 grams of crystalline calcium chloride to 100 c.c. of culture. This applies to these three experimental bacteria, and probably to all the others.

The action of sodium salts.—I have previously found common salt to have rather a weak action, but as it occurs in most media, and is frequently quoted as a flocculating agent, it was used upon these experimental bacteria. There was no flocculation, however, with small quantities. Larger quantities gave a similar result even when the salt was added in proportions varying from 550 to 7,700 milligrams of sodium per 100 c.c. and the tests were allowed to stand for 24 hours. Since this is equal to nearly 20 per cent. of common salt, it is evident that sodium salts do not flocculate bacteria.

The action of potassium and ammonium salts.—Although the action of these is much superior to sodium salts, no flocculation was obtained, and in the case of potassium chloride even when 24 per cent. was present. It can, therefore, be said that these salts do not flocculate bacteria.

The action of peptone.—This is not recognised as a flocculating agent, but since bouillon cultures contain 1 per cent. and bacteria are generally found more or less precipitated in bouillon cultures, a trial with it seemed advisable. As no coagulation appeared even when the culture contained 10 per cent., its use as a flocculating agent may be discounted.

The effect of temperature.—It is a well-known fact that heating causes the rapid precipitation of many chemical precipitates. Extreme temperatures cannot be employed in working with living bacteria, and the range between room temperature and blood heat was found to be too narrow to show any difference in the precipitation of the cultures with calcium chloride.

The action of lime water.—Whilst the hydrates of potassium and sodium prevent the flocculation of inorganic particles, hydrate of calcium greatly assists flocculation. This fact was remembered when calcium chloride was chosen in these experiments. When a solution of calcium hydrate, containing 2 milligrams of calcium per c.c., was gradually added to a culture of *Bact. prodigiosum*, a precipitation occurred. This was complete in half-an-hour when a volume equal to the volume of culture had been added.

Several circumstances were noted in the lime-water experiments. The bacterial culture when neutralised to phenolphthalein with sodium hydrate, was still alkaline when an equal and corresponding quantity of calcium hydrate had been added. Taking 10 c.c. portions, neutrality was reached with 2·35 c.c. tenth-normal soda and 6·0 c.c. tenth-normal lime. Sterile bouillon made neutral to soda was found to be still alkaline to lime, and as the latter was gradually added a flocculent precipitate continued to appear until neutrality was reached. This curious behaviour of lime and soda suggested the presence of phosphates of the alkalies, a suspicion that was confirmed by testing the precipitate, which proved to be tricalcium phosphate. The alkaline phosphates are derived from the meat which forms the basis of the culture media, and these are not completely precipitated when the media are neutralised with soda.

It is evident that this raises the whole question of precipitation by calcium salts, since it is probable that the coagulum obtained in the experiments consisted of bacteria entangled in a matrix of calcium phosphate. This is all the more probable, since the precipitates were certainly more voluminous than could have been expected from a simple flocculation of bacteria. On the other hand, however, microscopical examination showed the bacteria to be aggregated into large clumps, which would not have obtained had they been simply caught in a coagulum. Again, the trapping of the bacteria in calcium phosphate does not explain the different sensibility of *Bact. typhi* as compared with *Bact. coli commune*. It is true that the cultures were differently acid, but this was not found to be so very marked, 10 c.c. of *Bact. typhi* culture being neutralised with 0·75 c.c., and the same volume of *Bact. coli commune* or *Bact. prodigiosum* culture with 0·90 c.c. tenth-normal soda. The relative acidity would probably have been more marked had traces of glucose been allowed to be present. The meat extract had been permitted to begin a spontaneous bacterial growth, and consequently the traces of glucose, that are generally present, had been eliminated.

To obtain a medium free from the disturbing influence of phosphoric acid, bouillon was shaken up with slaked lime for several hours and filtered; washed carbon dioxide was passed through the filtrate for some time and the precipitated carbonate filtered off. The filtrate was then boiled to decompose the dissolved bicarbonate and the fluid again filtered to separate the precipitated carbonate. The resulting neutral medium contained no phosphoric acid and no lime, which was shown by testing with ammonium molybdate and ammonium oxalate. Neither did it give a precipitate with calcium salts, even with the addition of small quantities of sodium hydrate.

The three experimental bacteria grew slowly in the phosphate-free bouillon. When they had made some headway portions were tested, and the bacteria were found to be entirely unaffected by the addition of calcium chloride, potassium, ammonium or sodium salts. When the phosphoric acid was restored to the medium by the addition of traces of potassium phosphate, calcium chloride resumed its flocculating power. On treating the phosphate-free cultures with calcium chloride and alkali (0·5 c.c. tenth-normal sodium hydrate to 2 c.c. culture) a fine precipitate was obtained which very slowly gravitated. The deposit when examined microscopically was found to contain no bacterial floccules, and the organisms were free in the supernatant liquid. Accordingly it seems probable that the precipitate was the calcium salt of an organic acid elaborated by the bacteria. The precipitate is more marked when the bacteria are killed and partly disintegrated by boiling.

These experiments have shown that a pure flocculation of bacteria by means of the usual flocculating agents cannot be obtained. The reason for this is undoubtedly because the salt diffuses quickly through the bacterial cell and no surface pressure is occasioned. In working with dilute solutions of calcium chloride or better calcium bicarbonate and cultures containing phosphates, a flocculation of the bacteria is noticed, and this is quite apart from the entangling action of the calcium phosphate. It is worthy of emphasis that the amorphous particles of recently

precipitated tricalcium phosphate are so like large clumps of bacteria that they might readily be mistaken for such. In a mixture of phosphate particles and bacterial clumps, differences are to be seen in the smaller clumps where the individual bacteria can be recognised. That nascent calcium phosphate should flocculate bacteria is to be expected from the fact that, as well as being non-diffusible, it has an affinity for loose water molecules, and forms with them hydrated calcium phosphate. Soon after formation it becomes less and less hydrated, and when added to cultures at this stage no flocculation of bacteria occurs.

We can now refer to the differing susceptibility of the three experimental organisms to the action of calcium chloride. Microscopical examination of the precipitates did not indicate anything unusual, because the precipitated calcium phosphate looked precisely like large clumps of bacteria. There are two causes that might be brought forward—(1) The bacteria which have the most flagella become sooner entangled in the tricalcium phosphate coagulum; and (2) the organisms may elaborate substances which are precipitated by the calcium salt. With regard to the first cause, it is to be noted that the amount of calcium necessary to produce a certain effect in the cultures is inversely proportional to the number of flagella on the organisms. The greater the number of flagella, the more firmly will the organisms be retained by the calcium precipitate, and thus less precipitate need be formed to ensure the complete trapping of the bacteria. Another indication might be taken from the observation that the bacteria when living are more sensitive to the action of calcium chloride than when dead. With living cultures complete precipitation was obtained with a certain quantity of salt in one hour, while with dead cultures the same effect was obtained in twenty hours. The steaming requisite to kill the bacteria would undoubtedly shrivel up the flagella.

The second cause may have much more to do with the phenomenon than the first. Among the chemical products of the bacteria there are acids, but since the production of acid was limited by the total absence of glucose in the experimental

media, the differences in the acid-content of the cultures were very small. Since acid in the culture would dissolve a certain amount of calcium phosphate, the culture that contained most acid would require the addition of most calcium chloride to produce a certain effect. The relative acidities of the cultures, however, were not sufficiently distinctive to account for the difference in the amounts of calcium chloride necessary for complete precipitation. The nature of the acid radicles in the culture will probably explain the chief reason of the differing susceptibility. That the bodies of the bacteria have only a small function in the phenomenon is to be seen from the behaviour of filtered cultures. Three cultures grown in ordinary neutralised bouillon were filtered through porcelain filters and 2 c.c. portions were treated with calcium chloride ($\frac{1}{10}$ gram-molecule per litre) clear supernatant fluids were obtained in one hour with the following amounts of solution in c.c.:—

Bact. typhi	0·4
,, *prodigiosum*	0·8
,, *coli commune*	1·0

The differences are sufficient to indicate that it is to the product of the bacteria that the phenomenon is due. I am of the opinion that the cause may be traced to *Bact. typhi* withdrawing less phosphoric acid from the medium than the other two organisms which take up more and replace what they have taken with other acids. These acid products of metabolism form with calcium, insoluble salts which have less tendency to coagulate into floccules than tricalcium phosphate.

The differing susceptibility of *Bact. typhi* and *Bact. coli commune* to calcium salts can be utilised to distinguish between them. The method consists in pipetting two c.c. of a two or three days' bouillon culture into a narrow test tube and adding one c.c. of calcium chloride solution containing one gram crystallised calcium chloride per 100 c.c. The mixture is shaken and allowed to stand for an hour. At the end of this time *Bact. typhi* shows a well-defined precipitate, and in an almost clear supernatant fluid several large floccules adhering to the walls of the tube. *Bact.*

coli commune, on the other hand, has an ill-defined precipitate and a very turbid supernatant liquid.

From these numerous experiments I have shown :—

1. That bacteria are not flocculated by salts of potassium, sodium or ammonium like particles of suspended inorganic matter, and consequently that a pure flocculation or coagulation cannot be employed as a means of separating bacteria from cultures or of causing ultra-microscopical bacteria to cohere into visible cell-aggregates.
2. That salts of lime form a precipitate of calcium phosphate with the phosphoric acid of the medium.
3. That, since all ordinary media contain phosphates, and the organisms grown therein always retain traces of phosphoric acid, any substance capable of forming an insoluble phosphate will, when added to bacterial suspensions, cause a precipitate to form, and this, by entrapping the bacteria, will produce an apparent flocculation of the organisms. Microscopical examination may not indicate the presence of a precipitate because some insoluble phosphates, as for instance tricalcium phosphate, appear like large bacterial clumps.
4. That bacteria when grown in ordinary media exhibit different powers of precipitation with calcium salts, *Bact. typhi* requiring only one-fifth the amount required by *Bact. coli commune*.
5. That calcium chloride can be employed as a means of distinguishing between these two organisms.